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ORIGINAL PAPER

A chemical-inducible Cre-*Lox*P system allows for elimination of selection marker genes in transgenic apricot

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Abstract Transgenic plant development relies on the introduction of marker genes along with the gene(s) of interest to select and/or identify transgenic regenerants. Due to public concerns and regulatory issues, it would be advantageous to eliminate these marker genes once they are no longer needed. The chemical-inducible Cre-*Lox*P system is especially suitable for clonally-propagated plants, such as fruit trees, as no sexual crosses or rounds of transformation are required for marker-gene elimination. In this study, four transgenic pX6-GFP apricot (*Prunus armeniaca* L.) (cv. Helena) lines, carrying the *gfp* reporter gene encoding for the green fluorescent protein, were obtained following *Agrobacterium tumefaciens*-mediated transformation of leaf explants. The DNA site-specific recombination was precise and tightly controlled by the inducer β-estradiol. Expression of the *gfp* gene was only detected when 3 μM β-estradiol was added to the medium. When nodal explants were incubated on a meristem development medium supplemented with 3 μM β-estradiol, marker gene elimination was observed in buds of all four transgenic lines, at an average frequency of 11.3 %, based on GFP expression. Further molecular analyses of four GFP-positive shoots, a single shoot from each transgenic line, revealed that DNA recombination was complete in two of shoots, but incomplete in the other two shoots.

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Introduction

Conventional breeding of temperate fruit trees is constrained by their long juvenile periods, requirement for large land areas, significant field costs, and the seasonal temperature fluctuations needed to promote dormancy, flowering, and fruiting. More recently, research has focused on marker-assisted selection as a means to advance fruit tree breeding. However, this strategy is still limited by the inherently-slow generation cycles, complex reproductive biology, and high degree of heterozygosis of fruit trees.

An important advantage of genetic transformation is that a single gene or a few genes can be transferred into the plant genome. These genes may originate from within the species or, for novel traits not available in the gene pool of the species, from other species. Once a desirable transformant is isolated, it can be grafted or cultured in vitro; enabling the unlimited production of the desired transgenic line. Regeneration and transformation systems using clonal material (as opposed to seed or embryo based systems) are needed for moving useful genes into specific cultivars.

Most plant transformation systems have relied on dominant, selectable marker genes based on herbicide or antibiotic resistance (Manimaran et al. 2011). Selectable marker genes are co-delivered with the gene(s) of interest in order to identify and encourage the growth of the rare cells that accepted the integration of foreign DNA. This is especially true in the case of woody fruit trees, where low transformation efficiencies are common (Petri and Burgos 2005). In most cases, selectable marker genes remain in the transgenic

plants after transformation (Manimaran et al. 2011). A reporter gene may also be added to the construct allowing the visual verification of transformation while growth proceeds on a selective medium. Beyond the initial selection and transformation authentication, marker genes serve no further purpose in transgenic plants and their presence could be problematic for commercial biotechnology products due to consumer concerns and regulatory requirements.

Over the past decade, researchers have developed several approaches for the regeneration of marker-free transgenic plants (Darbani et al. 2007; Manimaran et al. 2011). Of these, some require rounds of hybridization and segregation to create marker-free plants. This is not practical for fruit trees, such as *Prunus* spp., due to their long reproductive cycles and heterozygosity.

The use of site-specific recombination systems, such as the Cre-*Lox*P (Zuo et al. 2001) or multi-auto-transformation (MAT) (Ebinuma and Komamine 2001) vectors, may be more suitable for the generation of marker-free transgenic trees. The MAT vector system, that combines the *Agrobacterium* oncogene *ipt*, as a selectable gene and the R/*RS* excision system from *Zygosaccharomyces rouxii*, has been applied to the transformation of some tree species such as citrus (Ballester et al. 2007, 2008), apricot (López-Noguera et al. 2009), and poplar (Zelasco et al. 2007). More recently, the R/*RS* system has been applied in combination with a bifunctional, selectable marker gene for the generation of marker-free cisgenic apple plants expressing an apple scab resistance gene (Vanblaere et al. 2011).

Removal of DNA from plant cells by using the Cre-*Lox*P system from bacteriophage P1 was first reported by Dale and Ow (1990). The original method required sexual crossing to eliminate selectable marker genes (Dale and Ow 1991), which made it unsuitable for use with fruit trees. A significant improvement to the method was subsequently reported whereby the recombinase was placed so that it acted in *cis*. The recombinase expression was tightly controlled by an estrogen receptor-based fusion transactivator (XVE) to remove the marker and recombinase genes (Zuo et al. 2001).

Recently, regeneration of marker-free transgenic fruit trees using binary vectors devoid of a selectable marker gene has been described in apple (Malnoy et al. 2010), sweet orange (Ballester et al. 2010), and plum (Petri et al. 2011). However, in these cases, very-efficient regeneration/transformation protocols were available.

In our laboratory, an *Agrobacterium*-mediated transformation protocol for mature apricot tissues was developed using antibiotic selection (Petri et al. 2008a, b). More recently, this protocol was used in combination with a MAT vector (López-Noguera et al. 2009). Although we demonstrated that MAT vectors could be used for marker gene elimination in apricot plants, only 41 % of the "marker-free" shoots were GUS positive (the gene used in the experimental construct as the recombination event reporter). This suggested that anomalous recombination was taking place in this species, as demonstrated previously in other species such as citrus (Ballester et al. 2007) and barrel medic (Scaramelli et al. 2009).

Although the Cre-*Lox*P system has been used successfully for selectable marker gene elimination in the model tree hybrid aspen (Fladung and Becker 2010), here we report for the first time the use of the Cre-*Lox*P system to obtain marker-free transgenic plants in a fruit tree species.

Materials and methods

Transgenic plant regeneration

This study was carried out using the apricot cv. Helena. For plant transformation, the *Agrobacterium tumefaciens* EHA105 strain harboring the binary plasmid pX6-GFP (Zuo et al. 2001) was used. In this construct, the expression of the *Cre* recombinase is controlled by an estrogen receptor-based fusion transactivator XVE. Upon induction by β-estradiol, sequences encoding the selectable marker, *Cre*, and XVE sandwiched by two *Lox*P sites are excised from the construct, leading to activation of the downstream green fluorescent protein (*gfp*) reporter gene (Fig. 1), which represents the possible gene of interest in this experimental construct. The engineered *Agrobacterium* strain was cultured and prepared for explant infection as described in Petri et al. (2008b).

Transgenic plants were regenerated from leaf explants of in vitro shoots following previously-published procedures (Petri et al. 2008a). Briefly, the first four apical, expanding leaves from 3-week-old proliferating shoots were collected. Leaves were then incubated for 10 min in the bacterial suspension with gentle shaking. Incubated leaves were blotted dry on sterile filter paper, cut transversely three or four times across the midrib without fully separating the segments, and cultured with the adaxial side in contact with the co-culture medium. The co-culture medium was regeneration medium (RM) (Petri et al. 2008b) without silver thiosulfate and supplemented with 100 μM acetosyringone and 9.05 μM 2,4-dichlorophenoxyacetic acid. After the explants were positioned on the medium, the dishes were sealed with Parafilm and incubated in the dark at 22 ± 1 °C for 4 days before transfer to RM with 60 μM silver thiosulfate + 0.63 mM cefotaxime and 0.13 mM vancomycin, for *A. tumefaciens* growth control, and 10 μM paromomycin for the selection of transgenic plant tissues. After 10 days, the explants were transferred to the same medium with 40 μM paromomycin for the rest of the experiment. After 2 weeks in the dark, the explants were transferred to white light with a 16-h photoperiod and 55 μmol m^{-2} s^{-1} intensity.

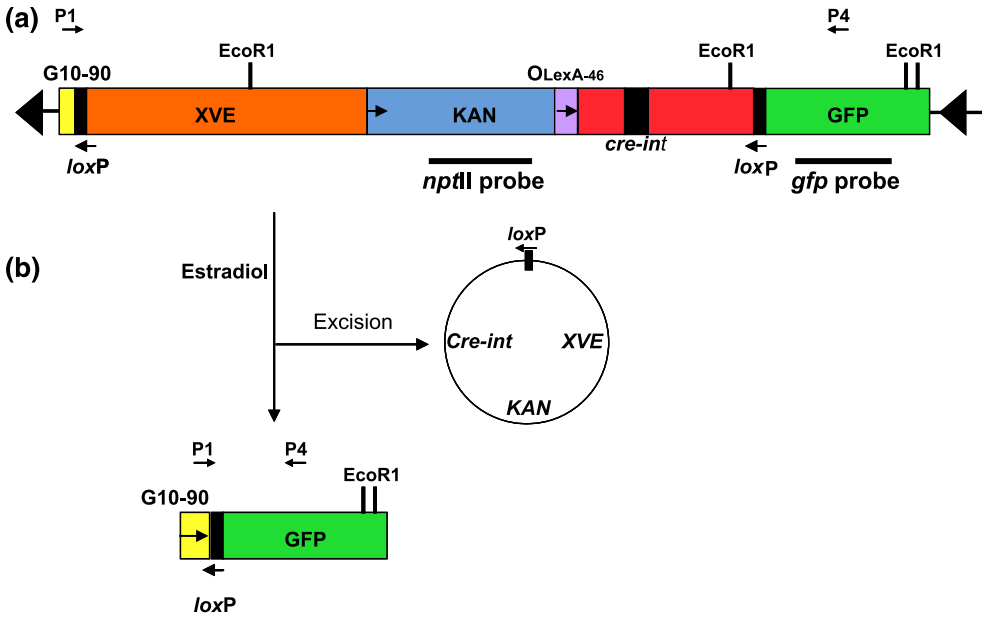

Fig. 1 Schematic representation of pX6-GFP plasmid T-DNA before (**a**) and after (**b**) the *β*-estradiol-induced site-specific DNA recombination. Three transcription units are located within the two *loxP* sites: *XVE* consists of the coding sequence of the XVE hybrid transactivator terminated by the *rbcs* E9 polyA addition sequence, and is activated by the *G10-90* promoter upstream of the *loxP* site; *KAN* consists of the nopaline synthase (*NOS*) gene promoter, the coding sequence of the neomycin transferase II (*npt*II), and the *NOS* polyadenylation sequence; *cre-int* consists of eight copies of the *LexA* operator sequence fused to the −46 CaMV*35S* promoter, the coding sequence of Cre interrupted by an intron, and is terminated by the *NOS* polyadenylation sequence. Downstream of the second *loxP* site, the *gfp* is terminated by the *rbcs* 3A polyA addition sequence. *Arrows* inside squares indicate the direction of transcription. The P1 and P4 primers (Zuo et al. 2001) used for PCR analysis are shown in Fig. 2. The *Eco*RI sites and probes position used for DNA blot analysis in Fig. 3 are indicated

Buds were isolated, transferred to a meristem development medium (MDM), and later elongated in the presence of 40 µM paromomycin following previously-published procedures (Pérez-Tornero et al. 1999). Elongated shoots were transferred to shoot multiplication medium (SMM) (Petri et al. 2008b) containing 40 µM paromomycin and maintained until enough material was available for DNA extractions.

Evaluation of transgenic shoots

DNA was isolated from leaves of in vitro shoots cultured under selection (40 µM paromomycin), following standard procedures (Doyle and Doyle 1987). Tissue from different leaves along each shoot was collected and then crushed in the buffer by means of Tissue-lyser® (Qiagen GmbH, Hilden, Germany). PCR was performed in a final volume of 25 µL containing 2 mM $MgCl_2$, 450 µM dNTPs, and 0.4 µM of the primers used for each gene (Table 1). The reaction conditions were 3 min denaturation at 94 °C, 34 cycles of 1 min at 94 °C, 1 min hybridization at the appropriate temperature for each primer pair (Table 1) and 1 min at 72 °C, followed by 5 min of additional extension at 72 °C.

For Southern blot analysis, 20 µg of *Eco*RI-digested DNA samples were separated on a 1 % (w/v) agarose gel and

Table 1 Specific primers used for the PCR amplification of the different genes

Gene	Primers 5′ → 3′	Annealing temperature (°C)	Expected product size (bp)
*npt*II	Km F:gat tga aca aga tgg att gc	52	696
	Km R:cca agc tct tca gca ata tc		
gfp	gfp F:cac tgg agt tgt ccc aat tc	60	678
	gfp R:tcc atg cca tgt gta atc cc		
*vir*G	VirG F:ccg ttg aaa cac gtt ctt	58	660
	VirG R:ccg gca cct ctt gct gtt tt		
DNA excision	P1[a]:cca tct cca ctg acg taa ggg at	60	909
	P4[a]:ttg tat agt tca tcc atg cca tg		

[a] As published by Zuo et al. (2001)

blotted onto positively-charged nylon membranes (Roche Diagnostics GmbH, Mannheim, Germany). The filters were hybridized with a Digoxigenin-11-dUTP alkali-labile (Roche Diagnostics GmbH, Mannheim, Germany) labeled probe coding for the *npt*II or *gfp* gene. Probes were prepared by PCR using appropriate primers (Table 1). Hybridization, washing, and detection were performed using DIG Easy Hyb (hybridization solution) and DIG Wash and Block Buffer Set, following the supplier's instructions (Roche Diagnostics GmbH, Mannheim, Germany).

Elimination of selection marker genes induced by β-estradiol

Cassette excision was first tested in transgenic apricot calli obtained after leaf explant infection. To ensure that the calli were uniformly transformed, they were subcultured every 2 weeks prior to cassette excision induction (in a medium described previously (Petri 2005) but with the addition of 172 μM kanamycin) until the calli were uniformly cream-yellowish without any brown areas. Chemical induction was performed by adding 3 μM β-estradiol to the medium (Zuo et al. 2000). At this point, no selection was applied. The β-estradiol was filter-sterilized with a 0.2-μm syringe filter and added to the medium after autoclaving.

In order to obtain buds where the excised cassette, nodal explants from in vitro-propagated transgenic pX6-GFP shoots were used. After the elimination of the axillary meristems, nodal explants were cultured in MDM (Pérez-Tornero et al. 1999) with the addition of 3 μM β-estradiol to induce marker gene elimination from the very immature pre-existing meristems in the nodal explants. The explants were cultured in the dark to reduce chlorophyll accumulation in the regenerated buds and facilitate GFP detection.

The evaluation of regenerated buds was performed by GFP detection. The calli and nodal explants were examined periodically under a Leica MZ75 stereomicroscope equipped with a fluorescence GFP Plus filter module, which contains a 480/40-nm excitation filter, a 505-nm LP dichromatic beam-splitting mirror, and a 510-nm LP barrier filter. A 50-W, high-pressure mercury vapor lamp provided illumination. The red autofluorescence from chlorophyll was not blocked with an interference filter.

The GFP-positive buds were isolated and proliferated, in the presence of 3 μM β-estradiol, for further molecular analyses: PCR, with the *npt*II and P1–P4 sets of primers (Table 1), and Southern blotting.

Results

Regeneration of pX6-GFP transgenic plants

Three independent experiments were performed each with 140 explants (420 total) (Table 2). The average percentage of infected explants that showed adventitious regeneration was low, 7.1 %, with only 1.3 buds per regenerating explant (Table 2). Adventitious buds started appearing 4 weeks after the beginning of the experiment and they stopped regenerating 4 weeks after. From a total of 38 regenerated buds (Table 2) only four survived the entire selection process and were propagated successfully in the selective medium containing 40 μM paromomycin.

All four lines were PCR positive for *npt*II (Fig. 2a), and PCR negative for *A. tumefaciens vir*G gene (not shown). No amplification product was observed in these samples using the P1–P4 set of primers prior to the addition of β-estradiol (Fig. 2a), indicating that the recombination event was not occurring. The integration of transgenes, *npt*II and *gfp*, into the plant genomes of all four lines was confirmed by DNA blot analyses (Fig. 3a, b). A hybridization signal was not detected in the negative control (DNA from a non-transformed 'Helena' shoot) with either of the DIG-labeled probes (Fig. 3a, b).

Chemical-induced cassette excision

The transgenic calli exposed to 3 μM β-estradiol were examined for GFP expression on a daily basis and GFP was clearly expressed (Fig. 4). GFP areas increased in number and size with the number of days of culture with the inductor (Fig. 4a–c).

Buds from nodal explants started appearing 2 weeks after the explants were placed in MDM with β-estradiol. The explants were examined for GFP twice a week. Although the explants were cultured in the dark to reduce chlorophyll synthesis (Fig. 4d), buds which regenerated

Table 2 Regeneration of transgenic apricot plants from in vitro leaf explants transformed with the pX6-GFP construct

Experiment	No. of explants	No. of explants with regeneration	Regeneration (%)	No. of buds	No. of buds per regenerating explant
1	140	6	4.3	7	1.2
2	140	13	9.3	13	1.0
3	140	11	7.8	18	1.6
Total	420	30	7.1	38	1.3

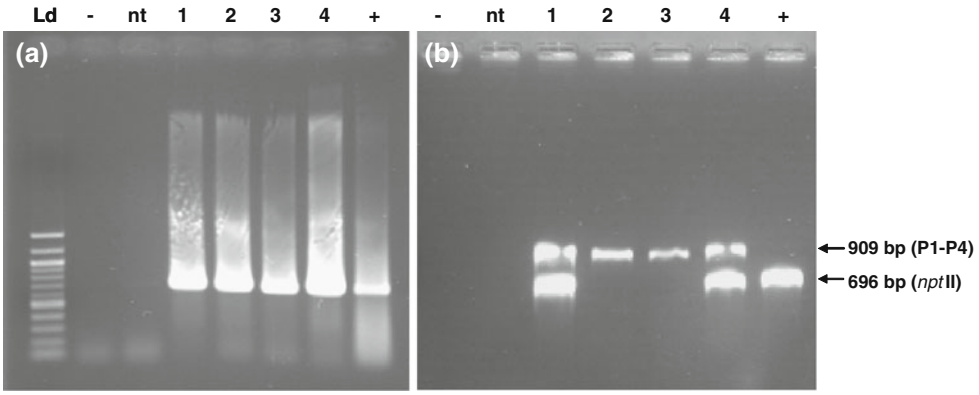

Fig. 2 Analysis of the PCR products of putative transgenic pX6-GFP apricot lines obtained with specific primers for the *npt*II gene or with the set of primers P1–P4 (Zuo et al. 2001) for cassette excision confirmation before **(a)** and after **(b)** *β*-estradiol-induced excision. *Lane Ld*: DNA ladder 100 bp (New England Biolabs, Cat. no. N3231S). *Lane* -: H_2O. *Lane nt*: Non-transformed 'Helena' shoot. *Lane 1–4*: Putative transgenic 'Helena' lines. *Lane* +: pX6-GFP plasmid

from cells where excision had not occurred appeared red under the stereomicroscope due to chlorophyll autofluorescence (Fig. 4e). Expression of GFP was detectable in some regenerated buds (Table 3; Fig. 4f). Regeneration of induced-GFP-positive buds was achieved from nodal explants of all four transgenic lines (Table 3). Four weeks after the nodal explants were placed in MDM with 3 μM *β*-estradiol, nine GFP-positive buds were obtained out of a total of 80 regenerated buds (Table 3; Fig. 4f). This represents an average of approximately 11.3 % regeneration of buds where site-specific recombination had occurred.

GFP-positive buds regenerated from nodal explants of all four transgenic lines were isolated, elongated, and proliferated. One shoot from each line was selected for further molecular analyses. As the buds developed and elongated, GFP expression was masked by the red chlorophyll fluorescence. PCR performed with the P1–P4 set of primers (Table 1) produced the 909-bp band expected after DNA recombination in the GFP-positive shoots (Figs. 1b, 2b). Sequencing of the 909-bp PCR product revealed that the recombinase acted precisely at the *Lox*P site (data not shown). Southern blot analyses of the *Eco*RI-digested DNA of the four plants confirmed that recombination had occurred in all four tested plants. Nevertheless, total excision had only occurred in two plants, where the *npt*II signal was not detected and the *gfp* probe hybridized with a band of different size from the one generated before DNA recombination (Fig. 3c, d: Lanes 2 and 3). Plants where total excision had not occurred yet (with cells where CRE recombinase had acted and cells where it had not) still showed a signal with the *npt*II probe (Fig. 3d: Lanes 1 and 4). The hybridization pattern with the *gfp* probe showed the fragment before excision plus an additional band (Fig. 3c: Lanes 1 and 4). These results suggest that each transgenic line had a single insertion of the construct in the genome (Fig. 3c).

Discussion

Although *npt*II has been considered a safe selectable marker gene by the EFSA (E.F.S.A. 2004), public concern, particularly in Europe, about antibiotic-resistance genes in genetically-modified plants makes the elimination of marker genes important if a final commercial product is desired. This may ease the path to crop release and commercialization, in terms of time and costs.

Over the years, the Cre-*Lox*P system has been proven to be an efficient and precise tool for marker gene elimination by plant biotechnologists (Gilbertson 2003). Detailed studies have confirmed the precision of the CRE recombinase (Sreekala et al. 2005; Zhang et al. 2003, 2006). The construct used in our study (Zuo et al. 2001), where the recombinase acts in *cis*, makes the system suitable for fruit tree biotechnology since sexual crosses are not required for selectable marker gene elimination. Another advantage of the construct used here is that its expression is tightly controlled by an estrogen receptor-based fusion transactivator XVE. Induction by *β*-estradiol removes both the selectable marker and recombinase genes. Constitutive high expression of the *Cre* gene in some plant species has been related to anomalous phenotypes such as chlorosis, curly leaves, or delayed growth and flowering (Coppoolse et al. 2003). Since DNA recombinases bind DNA, their high accumulation could interfere in ordinary DNA processes (Ow 2001). Studies in mouse cells showed that high *Cre* gene expression produced abnormal chromosome rearrangements (Schmidt et al. 2000), indicating that it is advisable to eliminate or inactivate the *Cre* recombinase gene as rapidly as possible. The transgenic lines obtained in our study showed normal in vitro phenotypes. In the future, further studies could be performed in order to determine their ex vitro growth and flowering.

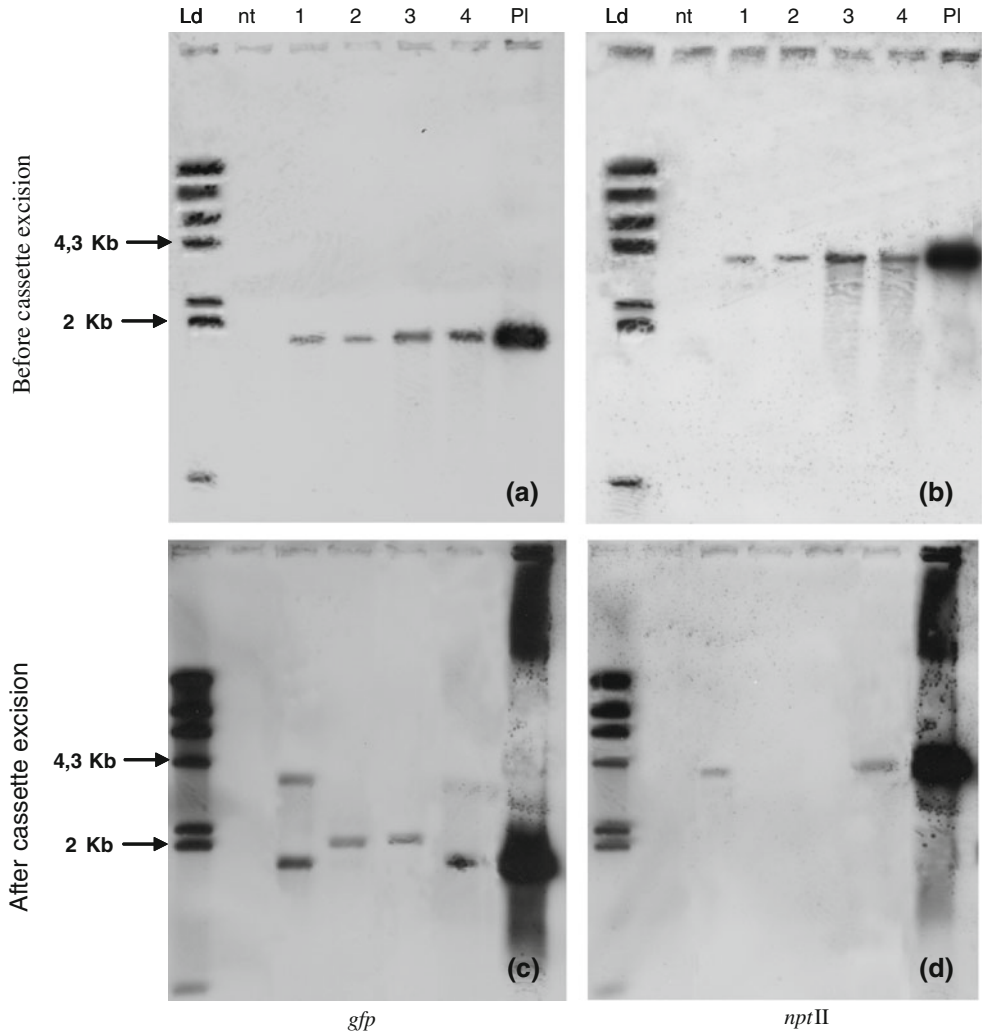

Fig. 3 Southern blot analyses of *Eco*RI-digested genomic DNA of transgenic lines. **a** Hybridization pattern with a *gfp*-labeled probe prior to the *β*-estradiol-induced, site-specific DNA recombination. **b** Hybridization pattern with an *npt*II-labeled probe prior to the *β*-estradiol-induced site-specific DNA recombination. **c** Hybridization pattern with a *gfp*-labeled probe after the *β*-estradiol-induced site-specific DNA recombination. **d** Hybridization pattern with an *npt*II-labeled probe after the *β*-estradiol-induced, site-specific DNA recombination. *Lane Ld*: DIG-labeled DNA molecular marker (Roche. Cat. no. 11218590910). *Lane nt*: Non-transformed 'Helena' shoot. *Lanes 1–4*: Transgenic apricot lines. *Lane Pl*: *Eco*RI-digested pX6-GFP plasmid

Table 3 Bud regeneration from nodal explants of transgenic apricot lines in medium containing *β*-estradiol

Line	No. of explants	Regeneration (%)	Total no. of regenerated buds	Buds GFP+	Frequency of GFP + buds (%)
1	18	88.9	21	2	9.5
2	20	80.0	30	1	3.3
3	14	42.9	12	3	25.0
4	10	80.0	17	3	17.6

Data were recorded 4 weeks after the nodal explants were placed in regeneration medium with 3 μM *β*-estradiol

After infection of 420 apricot leaves, four independent transgenic pX6-GFP lines were obtained. This represents approximately 1 % transformation efficiency. Our previous results using this same selection strategy showed a transformation efficiency of up to 1.3 % based on PCR analyses of surviving shoots (Petri et al. 2008b).

Southern blot analyses after cassette excision revealed that all four lines had a single insertion of the *gfp* transgene

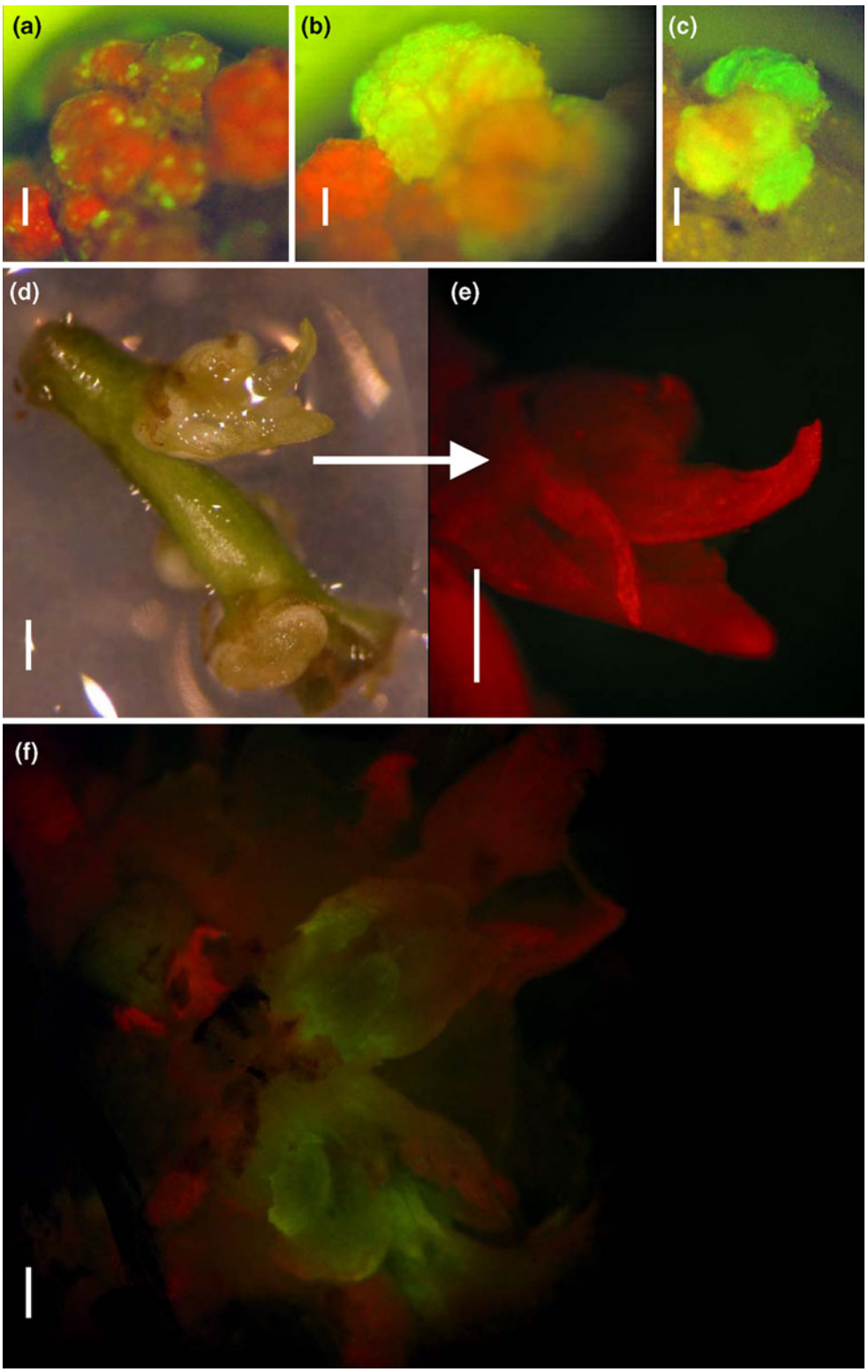

Fig. 4 GFP detection after β-estradiol-induced DNA recombination in plasmid pX6-GFP in transgenic calli after **a** 3 days, **b** 10 days and **c** 15 days exposure to 3 μM β-estradiol. **d** Nodal explant, from a pX6-GFP transgenic apricot line, cultured in the dark for 4 weeks in the presence of 3 μM β-estradiol with an etiolated adventitious bud. The photograph was taken with white light. **e** The same adventitious bud examined under blue light for GFP detection. Marker gene excision had not occurred and GFP was not detected. The *red color* corresponds to the chlorophyll autofluorescence under the blue light. **f** GFP detection in buds regenerated from a transgenic line nodal explant after 4 weeks culture in the dark with 3 μM β-estradiol. *Vertical bars* represent 1 mm. (Color figure online)

(Fig. 3c). These results, together with previously-published findings (Petri et al. 2008a, b), suggest that low copy number insertions into the apricot genome are common with this procedure.

The *gfp* marker gene was a useful reporter for site-specific recombination screening. This was particularly true when transgenic calli were examined. GFP was detected in induced transgenic calli after a few days of exposure to β-estradiol (Fig. 4a, b). After 15 days of exposure to the inducer, GFP was detected throughout the transgenic calli (Fig. 4c) indicating that cassette excision was induced in apricot. Since no selection was applied at this stage, GFP-negative and GFP-positive tissues should proliferate in a similar manner. In this case, the GFP-negative and GFP-positive areas should increase with the time of culture. However, in our case, only an increase in the GFP-positive areas was observed, suggesting that it was due to the action of the inducer, rather than the proliferation of GFP-positive callus cells. GFP was not detected at all when tissues were not exposed to the inducer (not shown). These results, together with the fact that the PCR amplification product was not obtained with the P1–P4 primers prior to the addition of β-estradiol, confirm that the site-specific recombination by CRE was tightly controlled by β-estradiol in apricot. This findings are consistent with previous reports in *Arabidopsis* (Zuo et al. 2001), tomato (Zhang et al. 2006), and rice (Sreekala et al. 2005).

In the regenerated buds, GFP was only detected in the early stages of development of etiolated buds (Fig. 4f), being later masked by the chlorophyll autofluorescence. To eliminate the possibility that this loss of GFP fluorescence was due to the lack of the transgene expression as the buds developed, RT-PCR was performed using RNA isolated from the buds where GFP was detected in the first steps of development and later lost. The results revealed the presence of *gfp* mRNA in all samples (data not shown). These results agree with previous studies in apricot where GFP was readily detected in transgenic calli and roots but frequently masked in green tissues (Petri et al. 2004, 2008a, b). The expression of GFP can vary for several reasons (Hraska et al. 2008). Moreover, autofluorescence of the plant tissues, generally a species-specific phenomenon, often leads to low signal-to-noise ratios (Billinton and Knight 2001). GFP was not effective for the screening of transgenic plum plants (Petri et al. 2011) but it has been described as a useful reporter gene in other woody plant species such as citrus (Ghorbel et al. 1999; Xu et al. 2011) or peach (Pérez-Clemente et al. 2004). In contrast, in another study in peach, the same *gfp* construct was poorly detected (Padilla et al. 2006) highlighting the variability reported by Hraska et al. (2008).

The molecular analyses (PCRs and DNA blots) coupled with the GFP detection confirmed that marker gene excision had occurred in all four transgenic lines after induction. This chemical-inducible, site-specific DNA excision seems to be species-related. In *Arabidopsis*, DNA excision occurred in all 19 transgenic lines obtained, at a frequency between 29 and 66 % of the germ cells, depending on the line (Zuo et al. 2001). In contrast, in rice, DNA recombination after exposure to β-estradiol occurred in only 10 lines out of 86 (Zhang et al. 2006).

Although marker gene elimination was confirmed in the shoots of all four apricot lines, DNA recombination was partial in two of them. These lines had cells where excision had occurred and cells where it had not. Such incomplete DNA excision has been reported before (Schaart et al. 2004; Zhang et al. 2006; Zuo et al. 2001). One way to reduce the appearance of chimeras may be the use of a bifunctional selectable marker gene as proposed by Schaart et al. (2004). In our laboratory, we are currently studying the application of this site-specific recombination system coupled with the *dao*1 selectable marker gene which allows a positive selection before DNA recombination and a negative selection after excision. This may enable marker-free plant recovery by simply changing one component in the medium (unpublished data).

We have shown the possibility of obtaining marker-free transgenic apricot plants with the chemical-inducible Cre-*Lox*P system at a reasonable efficiency. Other methods have been described for the production of marker-free fruit trees (without the requirement for sexual crossing or repeated transformations), such as the R/*RS* excision system (Ballester et al. 2007; López-Noguera et al. 2009; Vanblaere et al. 2011). Additional approaches have been reported which omit the use of a selectable marker. In this case, PCRs were performed to identify the transformed shoots but the applicability of this approach relied on high-transformation efficiencies (Ballester et al. 2010; Malnoy et al. 2010; Petri et al. 2011).

In future experiments, *gfp* could be replaced by a gene of interest in order to obtain marker-free, transgenic apricot plants. Some constructs have been shown to confer resistance to plum pox virus (Hily et al. 2007; Nicola-Negri et al. 2010), a major disease in stone fruit species, and it would be useful to obtain marker-free apricot plants with this trait.

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